

THYROID AXIS INHIBITION IN XENOPUS LAEVIS: GENE EXPRESSION CHANGES IN THE BRAIN.

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Abstract

Previously, we have proposed an amphibian-based thyroid axis screening assay in which NF stage 54 tadpoles are exposed for 14 day and disruption of the axis is determined by measuring developmental rate and thyroid morphology. Using this approach we have conducted experiments establishing comprehensive dose responses for three model thyroid axis antagonists methimazole, 6-propylthiouracil, and perchlorate. In the current experiments we have examined gene expression changes in the brain following exposure to these inhibitors using cDNA and oligonucleotide arrays specific to *Xenopus laevis*. Stage 54 tadpoles were exposed to methimazole (25 mg /L), 6-propylthiouracil (20 mg/L) and perchlorate (1000 ug/l) for 24, 48, or 96 hr, the brains were removed and processed to gene array analysis. We have previously shown that these chemical concentrations have comparable impacts on both developmental rate and thyroid gland morphology. A couple of the most interesting changes observed include those with myelin basic protein and myelin proteolipid protein. Both genes were up regulated by all three inhibitors in a time dependent fashion. These inhibitors are known to have their direct action on the thyroid gland and it is likely that these changes observed in the brain are a consequence of decreases in circulating T4, rather than a direct action of the chemicals. From these experiments we conclude that these genes show promise as biomarkers of thyroid axis inhibition in developing amphibians. *This abstract does not necessarily reflect EPA policy.*

Methods

Chemical Exposures

Stage 54 tadpoles were exposed under flow-through conditions to Lake Superior water, perchlorate (1 mg/L), methimazole (25 mg/L) or PTU (20 mg/L). Animals were remove at 1d, 2, 4d (for gene expression analysis, 8 d (thyroid histology), and 14d (developmental rate analysis). For developmental studies tadpole were grown in clean water until the desired stage was reached

Oligo Array

Membrane Preparation

•Oligos were designed using Array Designer 1.1 (PREMIER Biosoft International) Oligos selected near 3' end, 65-75 nt in length, Tm of 65 to 75 °C. Oligos were diluted to 1 nmolar, spotted in duplicate using pins slotted to deliver 1 microliter, and UV-crosslinked to membrane.

•Probe Preparation

•Total RNA extracted with Tri-Reagent (Sigma), Synthesized cDNA from 2 micrograms of total RNA with the MessageAmp™ aRNA kit (Ambion), using an oligo d(T) primer with T7 promoter, cDNA was transcribed with T7 RNA polymerase and biotinylated nucleotides (Enzo) to generate amplified antisense RNA.

Hybridization

•Membranes were incubated at 50 °C overnight with 15 to 25 µgs of amplified biotinylated RNA in a formamide-based hybridization solution. Membranes were washed 2X SSC at RT for 5 min, follow by two 15 min washes in 0.1X SSC at 50 °C

Detection

•Biotinylated probes were detected by chemiluminescence with the BrightStar BioDetect™ kit (Ambion)

cDNA Array

Probe Preparation and Hybridization

Total RNA was isolated from *Xenopus laevis* tadpole brain using TRIzol and 1 µg total RNA was used to make aRNA that was converted to radiolabeled cDNA that was used to hybridize to MAGEX cDNA arrays (Crump et al, 2002). Three independent replicates of 3-4 animals each were done.

Detection

The images obtained from a phosphorimager were analyzed using ScanAlyze version 2.5 (Eisen et al, 1998).

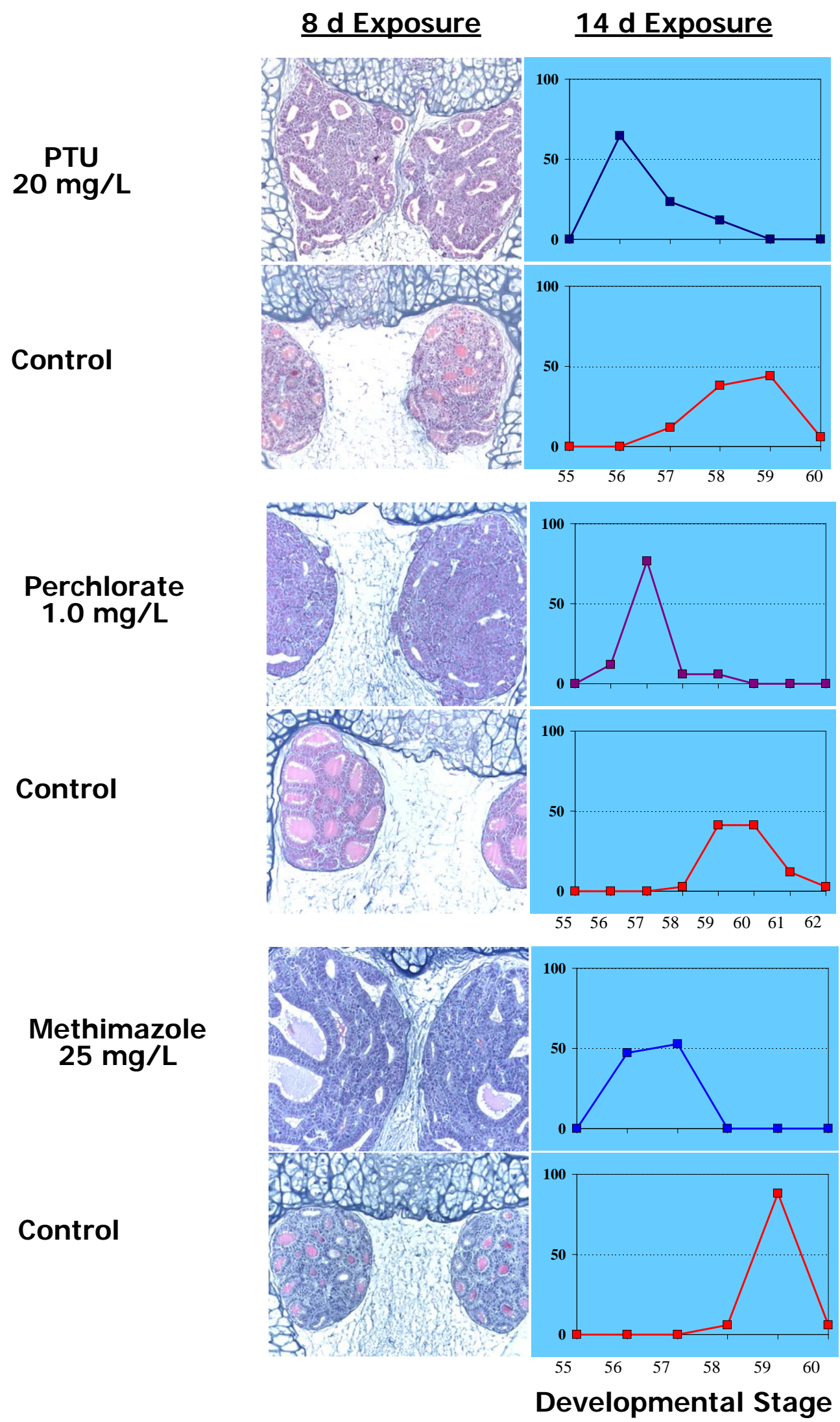
Data analysis

Relative expression values for each gene were determined from the median signal intensities across replicate array membranes for each treatment time point. Fold change relative to control for each treatment was then determined for each gene. The fold ratios were imported into Cluster 3.0 (Eisen *et al.*, 1998; de Hoon *et al.*, 2004) and log-transformed before being filtered for values greater than or equal to 1.0 in at least one observation (corresponds to 2.0-fold change). The centered data were then subjected to average linkage clustering to produce a hierarchical cluster tree in Java Treeview.

2D protein analysis

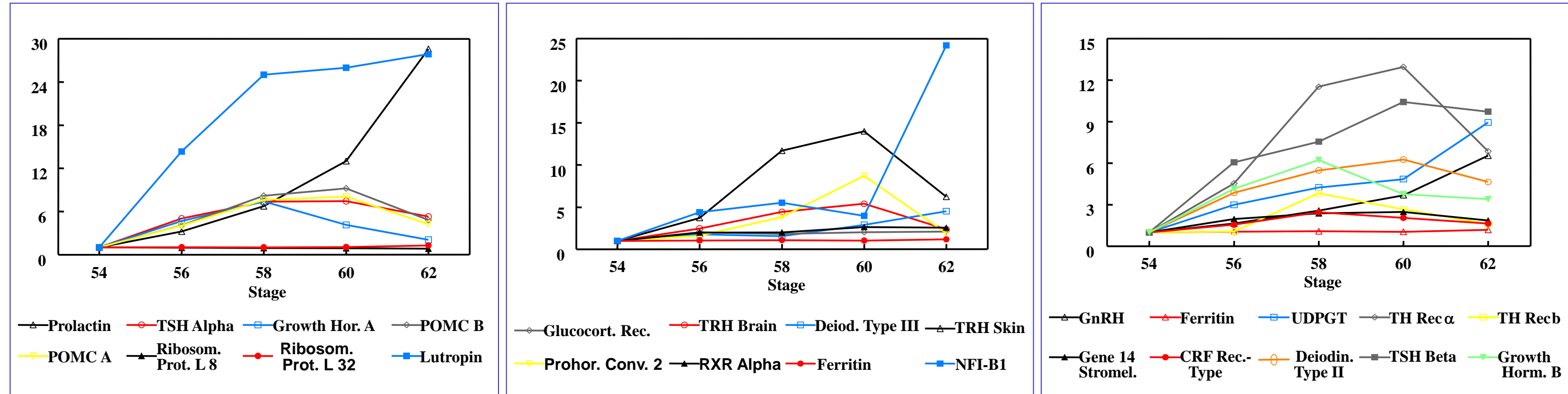
- Xenopus brain tissues (100 mg) were digested in 8M Urea, 4% CHAPS, 40mM Tris-HCl pH 9.5, 2mM TBP, 0.2% IPG buffer 3-10, 1mM Protease Inhibitor Cocktail and 1mM endonuclease.
- IEF was performed using Bio-rad's Protean Cell. IPG strips (11cm; 4-7 and 3-10 pH) were loaded with 190 uG of protein in Rehydration Buffer and focused for a total of 32000 V/h.
- Prior to SDS-PAGE, IPG strips were equilibrated first in 30% Glycerol, 6M Urea, 2%SDS, 2%DTT, 40mM Tris pH 8.8 and later in the same buffer without DTT, but iodoacetamide.
- 2D separation was run using Criterion 1mm 8-16% Gradient gels and the Bio-rad's Dodecacell.
- After electrophoresis gels were fixed, stained for 16h with SYPRO Ruby, and washed with water prior to imaging.
- Data analysis was performed using Bio-Rad's PD QUEST protein software.

Effects on Developmental Rate and Thyroid Histology

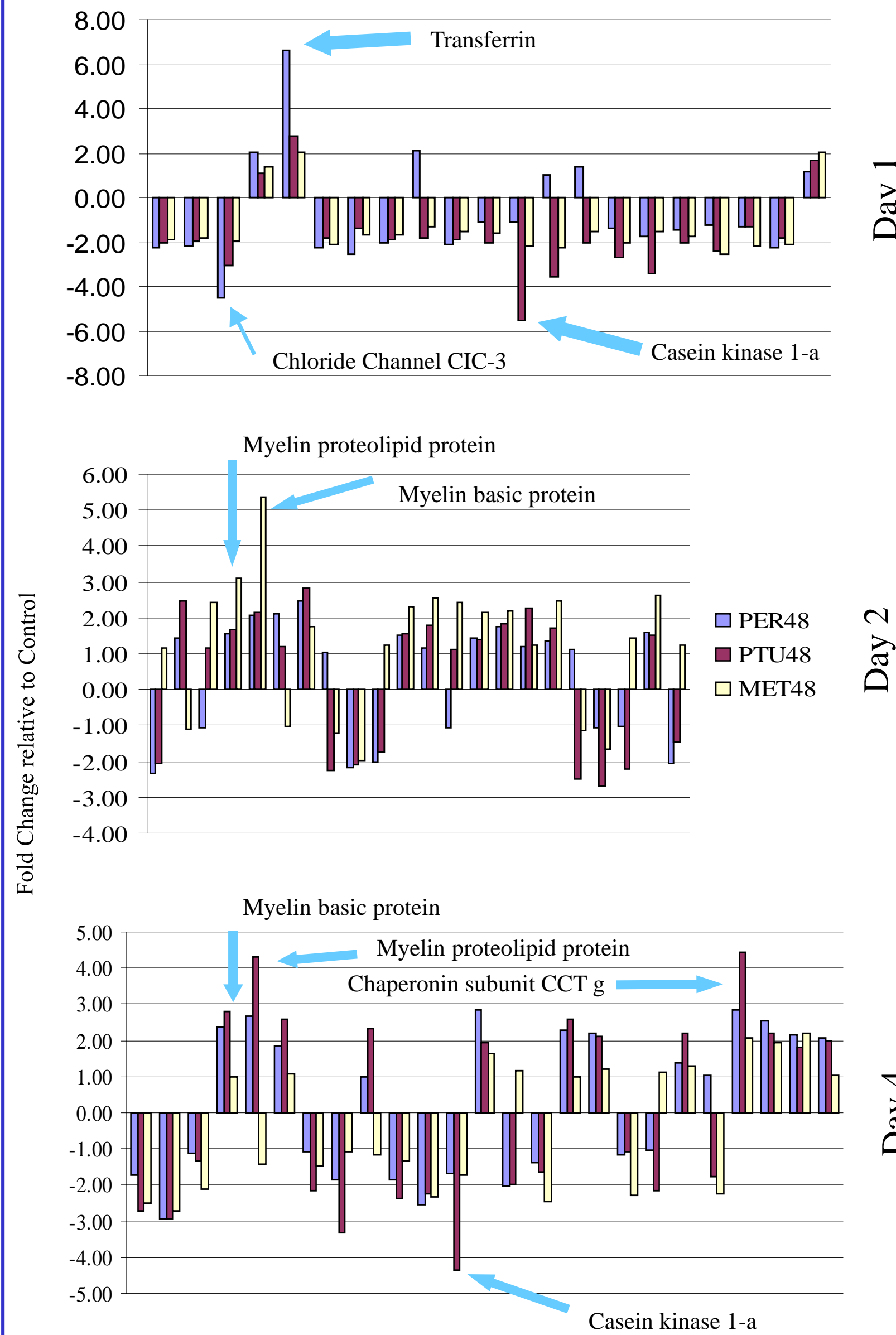


Gene expression change with developmental stage (relative to Stage 54) (Oligo Array)

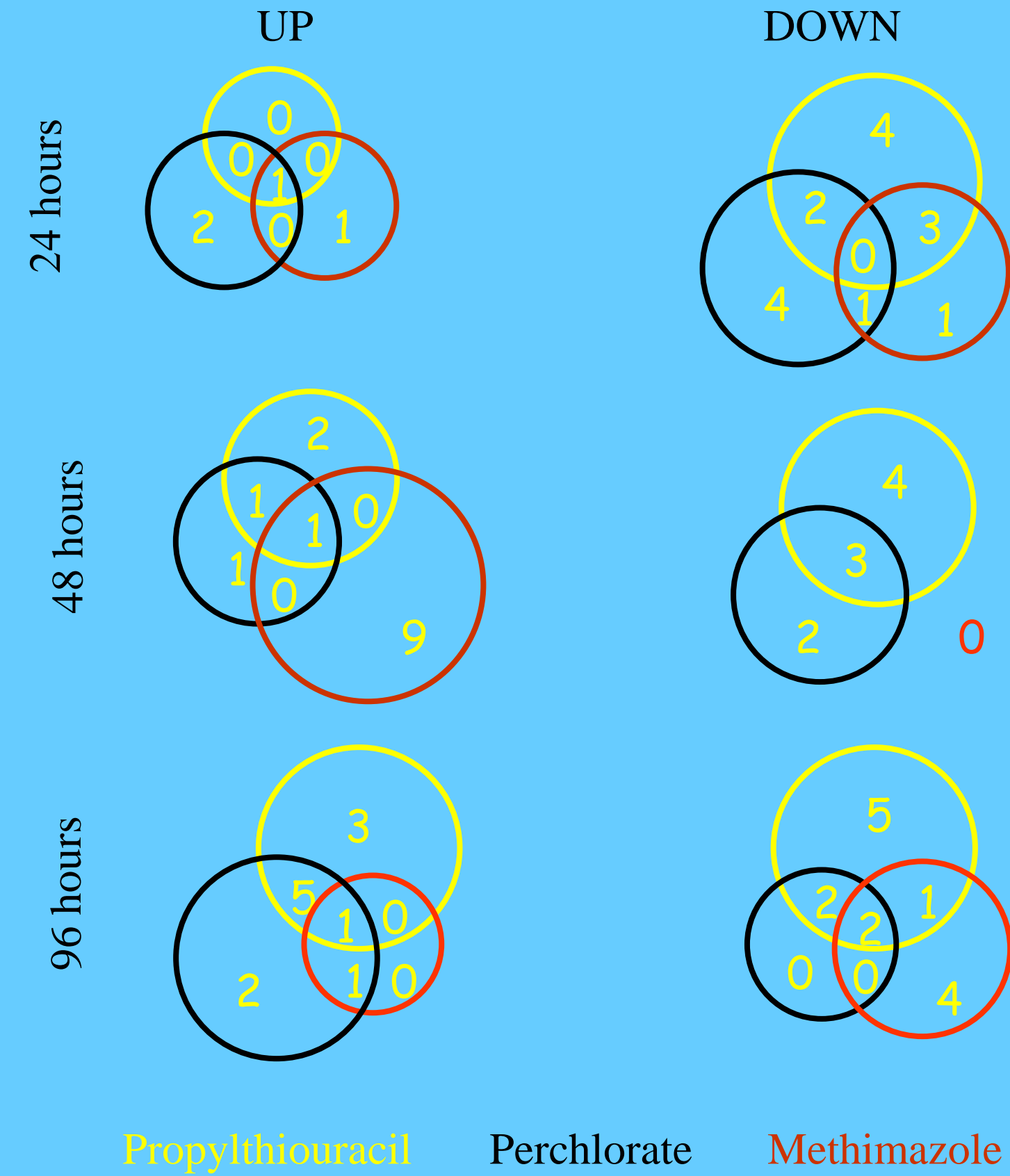
The figures below shows mean fold change at each stage compared to the mean of the stage 54 response, which was set to one.



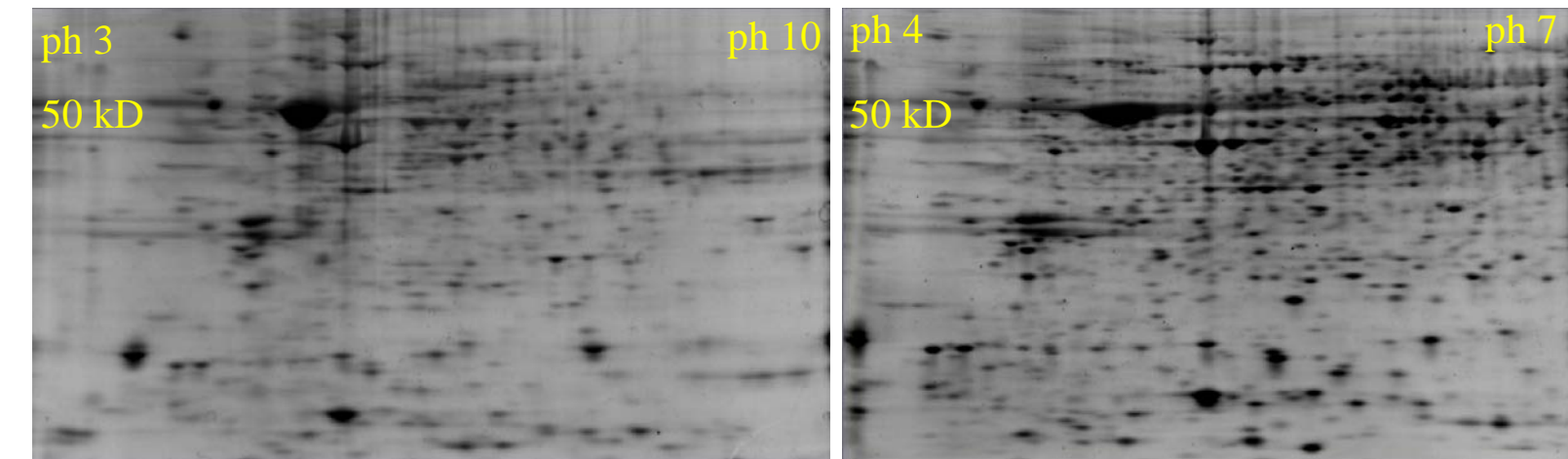
cDNA Array Results



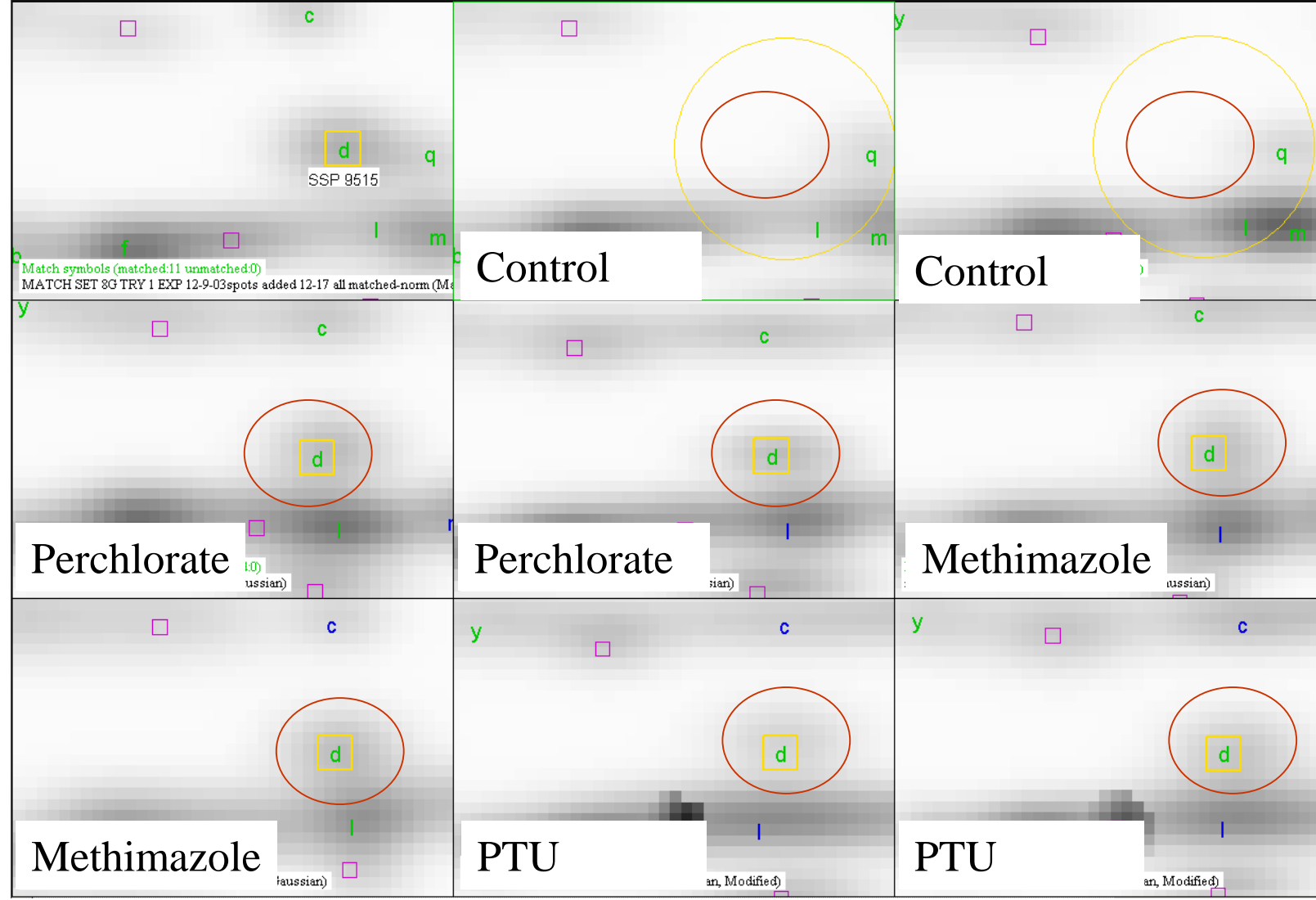
Transcripts that are altered at least 2-fold



Representative control stage 54 brains



11 proteins are up or down regulated in all treatment relative to controls. Below is an example of one such low expressed protein. MS characterization is currently in progress



Gene Name

Gene Name	Perchleate 24	PTU 24	Methimazole 24	Perchleate 48	PTU 48	Methimazole 48	Perchleate 96	PTU 96	Methimazole 96
protein/lipid phosphatase Pten									
negative regulator Id2									
TRIP4									
TRIP7									
coronin									
katanin p60									
myelin proteolipid protein									
myelin basic protein									
class II b-tubulin									
b tubulin									
a skeletal actin									
cytoplasmic b actin									
a-1 collagen type II									
serum retinol binding protein									
chloride channel CIC-3									
Ca2+-binding protein frequenin									
poly A-binding protein ABP-EF									
nervous system-specific RNA binding protein									
transferrin									
TIMP-2									
FGF-20									
growth hormone A									
PCNA									
MAP kinase phosphatase									
casein kinase 1-a									
cdk5									
14-3-3 protein									
natriuretic peptide receptor type C									
XICE-a									
CPP32									
ornithine decarboxylase									
lactate dehydrogenase A1									
GAPDH									
Na+/K+ transporting ATPase b subunit									
metallothionein									
convertase PC2									
aldolase C									
2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP1)									
arginase 1									
allantoicase									
liver helicase									
cytochrome b									
proteasome subunit Y									
transmembrane protein containing an EGF domain									
chaperonin subunit CCT g									
HMG-X									
calcineurin A									
nuclear factor 7									
proteasome subunit XC3									
B-50/GAP 43									

Conclusions and Future Directions

- Brain comparison showed stage-specific patterns of expression consistent with their playing a role in metamorphosis
- 2D gel analysis, although insensitive for lower abundance proteins, proves to be useful in identifying potential biomarker/diagnostic indicators of HPT disruption in amphibians
- Several genes (TRIP4, Myelin proteolipid protein, transferrin, chaperonin subunit cctg, nuclear factor 7, and MAP kinase phosphatase) were regulated similarly across all treatment and show promise of biomarker/diagnostic indicators of HPT disruption
- Future efforts will focus on QRT-PCR analysis of gene changes and MS characterization of protein changes